Quantitative Analysis of Gene Expression by Reverse Transcription Polymerase Chain Reaction and Capillary Electroporesis with Laser-induced Fluorescence Detection[1]

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Abstract

There has been a dramatic expansion of DNA sequence information compiled over the past several years for a variety of eukaryotic and prokaryotic genomes. Accompanying this increase in knowledge of genomic structure and organization has been a growing interest in studying the function of individual genes including regulation of their expression. A number of methods such as Northern blotting, ribonuclease protection assay, and hybridization arrays have been developed to analyze gene expression at the transcriptional (mRNA) level. Although quantitative estimates of mRNA transcripts can be obtained from each of these methods, oftentimes they lack sufficient sensitivity or the methodology is too costly or too labor-intensive to be applied to the analysis of a large number of samples. The most sensitive method for analyzing gene expression at the mRNA level involves the combination of reverse transcription and polymerase chain reaction (RT-PCR). However, in order to provide accurate quantitative estimates of gene expression, a rapid and efficient method is required for separation and detection of the double-stranded DNA (dsDNA) products of RT-PCR. Recent advances in capillary electrophoresis with laser-induced fluorescence detection (CE/LIF) have made this method suitable for the automated analysis of large numbers of RT-PCR samples. An overview of the application of CE/LIF to quantitative analysis of gene expression by RT-PCR is presented along with selected protocols and examples. Both relative-quantitative (RQ) and quantitative-competitive (QC) approaches to RT-PCR are discussed in conjunction with the use of CE/LIF for rapid and accurate quantitative analysis of PCR products.

Index Entries: Gene expression; mRNA; reverse transcription; polymerase chain reaction; capillary electrophoresis; laser-induced fluorescence detection.

1. Introduction

In order to investigate gene expression and its regulation at the transcriptional level, it is necessary to accurately quantify specific mRNA transcripts ***(1)***. A number of methods have been devised to quantitatively analyze mRNA, including Northern analysis, ribonuclease protection assay (RPA), cDNA-based hybridization microarray techniques, and reverse transcription polymerase chain reaction (RT-PCR). Hybridization methods such as Northern blotting and RPA, although still widely used, are relatively insensitive methods for quantitative analysis of gene expression, especially with regard to detecting gene transcripts of low abundance. Expression (cDNA) array technology allows for simultaneous screening of many genes, but quantitative estimates of individual gene expression can be limited by the sensitivity of detection methods and the complexity of the analysis. RT-PCR, because of the remarkable power of DNA amplification by PCR, offers the most sensitivity and is particularly useful in the analysis of low abundance mRNAs or for the analysis of small amounts of RNA

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obtained from limited tissue or cell samples. Real-time RT-PCR, a kinetic technique, relies on cycle-to-cycle detection for quantitation of accumulating PCR products using simple fluorescence or fluorescence energy transfer methods ***(1)***. Although capable of providing very precise quantitative results with high sample throughput, real-time RT-PCR is not yet widely utilized for gene expression analyses because of the considerable costs associated with specifically designed reagents and instrumentation.

One of the most important aspects of RT-PCR-based analyses is the separation and accurate detection of the amplified double-stranded DNA (dsDNA) PCR products (amplicons). The most common method of analyzing PCR products involves separating a portion of the reaction mixture by agarose or polyacrylamide slab gel electrophoresis with ethidium bromide staining to detect the amplicons ***(2)***. There are several disadvantages associated with slab gel techniques including:

1. Gel casting, running, and handling can be costly, labor intensive, and are not readily automated.
2. A significant portion of the PCR sample is typically consumed by this mode of analysis.
3. Buffer and reagent consumption, and hazardous waste generated from the use of radioactive probes and ethidium bromide stain, can be considerable.
4. Quantitation requires additional steps and instrumentation for gel imaging and densitometry.
5. The linear dynamic range of detection for ethidium bromide-stained dsDNA by densitometry is quite limited and this must be taken into account when attempting to quantify amplicon levels by this method.

During the past decade, capillary electrophoresis (CE)-based techniques have been developed and refined for the analysis of dsDNA ***(3–7)***. CE analysis has a number of advantages over conventional slab gel separation techniques including:

1. CE instrumentation is fully automated with respect to sample injection, separation, on-capillary detection, data collection and post-run data analyses.
2. Because the separation is conducted in a narrow bore capillary tube that facilitates Joule heat dissipation, a higher field strength (voltage) can be employed resulting in enhanced resolution and shorter run times.
3. Very small amounts (nanoliters) of sample are required for each analysis, thus preserving more of the original sample for subsequent procedures, such as cloning or sequencing.
4. It is now possible (using fraction collection techniques) to recover specific amplicons separated by CE in a "ready-to-use" format for post-PCR applications such as cloning, sequencing, or re-amplification by additional PCR.

This review describes the application of CE to analysis of dsDNA products of RT-PCR. Selected examples of analyses and analytical protocols typically conducted in our laboratory using CE-based techniques are included to illustrate the potential of this technology for the quantitative determination of gene expression.

2. Capillary Electrophoresis Methodology

Table 1 summarizes information concerning selected parameters that are key to establishing a robust and reproducible technique for the separation and quantification of dsDNA using CE. Important advances have been made in a number of areas including capillary coatings, sieving polymer matrices, and high-sensitivity detection methods.

Because of nearly identical linear negative charge density at neutral pH and above, dsDNA molecules exhibit an electrophoretic mobility in free solution that is virtually independent of their molecular size ***(8)***. Therefore, a gel or sieving matrix (e.g., viscous polymer solution) is required to effect a separation of DNA molecules based on their molecular size. Because uncoated (bare fused silica) capillaries exhibit a strong electroosmotic flow (EOF) in the direction of the cathode when filled with buffer solutions above pH 7, they are not commonly used for DNA separations. Instead, capillaries treated with a specific interior surface coating to greatly reduce or completely eliminate EOF are routinely employed in the sepa-

Table 1. Selected Technique Parameters for the Analysis of RT-PCR Products by Capillary Electrophoresis

1. Capillaries:
   a. Untreated (bare fused silica): not frequently used
   b. Coated: polyacrylamide, polysiloxane (e.g., DB-1®, DB-17®, µSil-DNA®), polyvinyl alcohol, polyethylene oxide
2. Separation Matrices:
   a. Buffer: 89-100 m*M*Tris/Boric Acid, 2 m*M*EDTA, pH 8.2-8.5 (1X TBE)
   b. Sieving Polymers/Gels:
      1. Chemical (Fixed) Gels: cross-linked polyacrylamide, bonded to capillary wall
      2. Replaceable Gels (entangled polymer networks): linear polyacrylamide, methyl cellulose, hydroxypropylmethyl cellulose, hydroxyethyl cellulose, polyethylene oxide, polyvinyl alco hol, agarose
   c. Fluorescent Intercalating Dyes: ethidium bromide, YOYO-1, YO-PRO-1, TOTO (thiazole orange; EnhanCE®, TO-PRO-1, TO-PRO-3, SYBR Green I®
3. Sample Injection:
   a. Hydrodynamic: high reproducibility; useful for quantitative analyses; direct injection of untreated samples is possible
   b. Electrokinetic: affected by sample salt concentration (i.e., Cl^-); prior dialysis or dilution of the sample is re quired
4. Detectors:
   a. UV: 254-260 nm, least sensitive
   b. Fluorescence
   c. Laser-Induced Fluorescence (LIF): up to 1000 x more sensitive than UV

ration of dsDNA. In light of this reduction in EOF, most DNA separations are conducted in reversed polarity mode (i.e., cathode at the capillary inlet side). Capillary surface coatings can either be covalently bound to the surface or dynamically adsorbed to the wall. Examples of typical surface coatings include polyacrylamide, polysiloxanes (dimethyl and phenyl-methyl), cellulose derivatives, polyethylene oxide, and polyvinyl alcohol ***(3–7)***.

Early CE separations of dsDNA made use of capillaries in which a polyacrylamide gel was polymerized in and cross-linked to the wall of the capillary, producing what has been referred to as a fixed or chemical gel ***(3–7)***. Although such gels are capable of very high resolution due to a well-controlled pore size, they are not commonly used in PCR product analysis because of problems associated with air bubble formation and limited useful lifetime ***(7)***. One of the major advances establishing CE as a routine method for DNA analysis has been the development of replaceable sieving matrices (also referred to as entangled polymer networks) that can be pumped into the capillary prior to initiating a separation and flushed from the capillary after the separation is complete. With this system of replaceable polymer solutions, a new "gel" is utilized for each sample separation. Some of the most widely used replaceable polymers are linear polyacrylamides, hydroxyalkyl- or akylcelluloses, polyethylene oxide, agarose, and polyvinyl alcohol ***(3–7)***. These compounds are employed to produce viscous buffered solutions that enable dsDNA to be separated based on molecular size ***(7,9)***. In general, manipulating the polymer length and concetration affects the resolving capacity for dsDNA molecules. Resolution can also be affected by the choice of a specific polymer, the ionic strength of the buffer, as well as the length and diameter of the capillary used to separate the dsDNA ***(9)***. Sieving buffers containing polymers such as hydroxyethylcellulose or hydroxypropylmethylcellulose can be pumped into the capillary at relatively low pressures (,20 PSI), whereas higher viscosity polymer solutions, such as those

containing linear polyacrylamides, require much higher pressures (approx 100 PSI) to move them through the capillary ***(10)***. For this reason, low viscosity sieving buffers are more widely used in dsDNA separations on commercial CE instruments. They also have the added advantage of producing much shorter run times than higher viscosity solutions. However, resolution is better with the higher viscosity polymer solutions and these sieving matrices may be useful when attempting to resolve PCR products that differ in size by a small (e.g.,,10–20 bp) amount ***(7,9)***.

Although most CE-based separations of PCR products utilize a sieving buffer, some attempts have been made to separate dsDNA using a simplified approach. For example, Liu and Chen ***(11)***recently reported a rapid (<3min) CE-based separation method for PCR products based on small differences in the charge-to-mass ratio of different sized dsDNA amplicons. Their technique employs a high pH buffer with ethidium bromide in an uncoated capillary without the addition of any sieving matrix. The EOF generated in the capillary was exploited to produce very rapid separation times. In fact, the shortened run time was suggested to be an advantage of this approach for screening large number of samples such as might be generated by a clinical laboratory. However, since the PCR product peaks (244 and 307 bp) were not baseline resolved, application of their technique to routine analysis of dsDNA is limited to the analysis of amplicons that are sufficiently different in size to permit their resolution. Huang et al. ***(12)*** obtained rapid DNA separations by CE in the presence of EOF. Their approach was to use an uncoated capillary with mixed hydrophilic polymer solutions and a high concentration of Tris/borate buffer. In this case the DNA molecules actually migrate counter to EOF.

One factor that initially hampered the direct analysis of PCR samples by CE is the presence of high levels of salt, especially chloride ions, in the samples injected into the capillary. Electrokinetic injection, which is the requisite loading method when using fixed gels, is severely affected by the presence of high salt concentration because it impairs the loading of dsDNA into the capillary. Although PCR samples can be directly loaded into capillaries with replaceable polymer solutions using hydrodynamic (pressure) injection, the presence of salts can adversely affect the separation. Also, the presence of other components in the PCR sample (dNTPs, primer oligonucleotides, and so on.) can also obscure product peaks when a less sensitive mode of detection such as UV absorbance is employed. Fortunately, two relatively simple methods have been devised to mitigate the effects of the PCR sample matrix. The two most common methods are sample microdialysis and dilution of the sample (20–100-fold) with deionized water prior to injection ***(5,13)***. Both methods are effective in reducing the adverse impact of salts, but sample dilution necessitates the use of a high-sensitivity detection technique to compensate for the reduction in the final concentration of dsDNA. An additional benefit of reducing the ionic strength of the sample is that it facilitates "stacking" of the negatively charged dsDNA molecules during electrokinetic loading. Thus, more dsDNA is loaded during injection and detection sensitivity is subsequently enhanced.

Another important development in CE technology that has helped to promote its use in the analysis of dsDNA is the introduction of laser-induced fluorescence (LIF) detection ***(14)***. Because LIF can increase the sensitivity of detection for dsDNA by 2–3 orders of magnitude over UV detection, it has quickly become the detection method of choice for the vast majority of DNA analyses ***(4)***. A practical illustration of the power of LIF detection is that typical separations of PCR products by slab gel electrophoresis with ethidium bromide staining require approx 5 ng of DNA per band for adequate detection, whereas, with CE/LIF sub-picogram levels of DNA are readily detected ***(5)***.

In order to employ LIF detection, it is necessary to label the dsDNA molecules with a fluorescent compound prior to and/or during their separation. Two approaches have been employed. The first, and by far the most common, involves incorporation of an intercalating dye into the separation

buffer (and in some cases into the sample loading buffer) that is highly fluorescent only when bound (intercalated) to dsDNA. **Table 1** lists a number of commonly used intercalating dyes. Both monomeric and dimeric dyes have been developed and successfully used to detect PCR products ***(15)***. A second approach involves labeling of the primers used in PCR with a fluorophore, such as fluorescein, to produce 5´-end-labeled dsDNA products. The former approach offers the highest sensitivity with the amount of intercalating dye bound being proportional to the size of the dsDNA fragment (i.e., the larger the fragment, the more dye will be bound). Not only do intercalating dyes label the dsDNA for detection, but they can also enhance the selectivity and resolution of dsDNA fragments of similar, or even identical, size because of the effects of dye intercalation on the rigidity of DNA double helix structure ***(5,6)***. However, intercalating dyes can produce anomalous effects on peak shape, depending on such factors as their concentration in the separation or sample loading buffers and certain sequence-dependent properties of the dsDNA molecule (e.g., %GC composition). These effects result from the binding and retention of variable amounts of dye molecules by dsDNA during CE. Therefore, care should be taken when choosing a specific labeling technique for a particular PCR product in order to generate the most accurate and reproducible results.

3. Capillary Electrophoresis and Quantitative RT-PCR

Quantitative analysis of gene expression requires an accurate determination of mRNA transcript levels ***(1,16)***. To accomplish this by RT-PCR, a complementary DNA copy of the mRNA (cDNA) is first produced by the process of reverse transcription (RT step). The PCR is then employed to amplify a double stranded form of that DNA copy to a level that can be readily detected (PCR step). Under optimal and controlled conditions, the amount of dsDNA produced by PCR is directly related to the quantity of mRNA present in the original RNA sample. **Table 2** summarizes some typical conditions and procedures that we have used to conduct RT and PCR for the purpose of analyzing the gene expression. RT-PCR can be performed in a single-tube or two-tube format, and there are numerous variations that can be employed to tailor both of these coupled reactions for the quantitative analysis of gene expression ***(16)***.

3.1. Capillary Electrophoresis: A Diagnostic Tool for RT-PCR

The first step in establishing quantitative RT-PCR analysis of gene expression is to optimize conditions for both reactions. The effects of adding differing amounts or types of RNA and reagents (enzymes, Mg^{2+}, dNTPs, oligonucleotide primers, and so on.) or using different reaction temperatures or times in the thermal cycling profile must be individually evaluated when analyzing the expression of a new gene by RT-PCR ***(16)***. The rapid separation and quantitation capabilities of CE/LIF make it an efficient technique to aid in the PCR optimization process by assessing the effects of changes in reaction conditions on PCR product quality and yield. **Table 3** lists key conditions and parameters currently employed in our laboratory for a CE-based technique designed for the rapid (5 min) separation and detection of PCR products ranging up to 1 kb in size. Integrated area (corrected for differences in migration time) is used to determine the quantity of each dsDNA product peak.

One important use of CE/LIF to evaluate PCR is to determine the amplification reaction kinetics. This is done by determining amplicon quantity at the completion of an increasing number of thermal cycles. **Figure 1** depicts a plot of integrated peak area vs cycle number for a ß-actin PCR product (300 bp) with the early, exponential, and plateau stages of the amplification indicated. Unlike ethidium bromide-stained agarose slab gel analysis, CE offers a much wider linear detection range that can be better utilized to define reaction kinetics, especially during the early stages of the reaction when the PCR product level is low and during the later stages of the reaction when significantly more product has accumulated. It is crucial for accurate quantitation in RT-PCR that samples be collected and analyzed within the exponential

Table 2. Typical Conditions for Reverse Transcription and Polymerase Chain Reactions

Parameter	Conditions
Reverse Transcription[1]	
1. RNA	1.0 µg of total RNA
2. Primers	100 ng random hexamers
3. dNTPs	500 µ*M*
4. Mg^{2+}	5 m*M*
5. DTT	10 m*M*
6. RNase Inhibitor	40 u
7. Reverse Transcriptase (RT)	Superscript II (50 u)
8. Total Reaction Volume	20 µL
9. Incubation Conditions:	a. Primer Annealing: 65°C for 5 min Place on ice for at least 1 min b. First Strand Synthesis: After adding 50 units RT incubate at: 25°C for 10 min 42°C for 50 min 70°C for 10 min c. RNase Treatment (After adding 2 u of RNase H): 37°C for 20 min. Place on ice or at -20°C
Polymerase Chain Reaction	
1. Mg^{2+}	2 m*M*
2. dNTPs	200 µ*M*
3. Primers (gene specific)	10 pmol each
4. *Taq*DNA Polymerase (Hot Start)[2]	1 u
5. RT Reaction	1 µL
6. Total Reaction Volume	25 µL
7. Thermocycling Conditions:	a. 1 cycle: 94oC for 2 min b. 30 cycles: 94oC for 30 s 58°C–60°C for 30 s 72°C for 1 min c. 1 cycle: 72°C for 8 min

[1] SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies, Rockville, MD).

[2] Platinum Taq DNA Polymerase (Life Technologies, Rockville, MD).

Table 3. Typical Conditions for Separation of PCR Products by Capillary Electrophoresis

Parameter	Conditions
1. CE System	P/ACE MDQ(r) (Beckman Coulter, Inc., Fullerton, CA)
2. Capillary	μSIL-DNA(r) (Agilent Technologies, Folsom, CA) 31 cm X 75μm ID (10 cm to detector)
3. Buffer	DNA Separation Buffer(r) (Sigma, St. Louis, Mo)
4. Intercalating Dye	EnhanCE(r) (Beckman Coulter, Inc., Fullerton, CA) Dilution: 0.5 μg/mL Separation Buffer
5. Detector	Laser-Induced Fluorescence: Excitation: 488 nm Emission: 520 nm
6. Temperature	Capillary maintained at 25oC via liquid cooling
7. PCR Sample	Diluted 1:100 in deionized water
8. Run	Methanol Rinse: 1.0 min at 20 PSI Buffer Rinse: 1.0 min at 20 PSI Injection/Water Plug: 5.0 s at 0.5 PSI Injection/Sample: 5-10 s at 3.0 kV Separate: 5.0 min at 8.0 kV

(also referred to as the linear) range of amplification during which each amplicon is accumulating at a constant rate ***(17)***. This ensures that the quantity of PCR product will accurately reflect the quantity of mRNA in the original RNA sample across a range of samples. CE is thus quite useful for accurately defining the exponential amplification range of PCR for a particular gene of interest.

CE analysis, because of its efficient resolution of dsDNA fragments, can also be used to accurately determine amplicon size. The size of a PCR product is defined by the unique set of primers used to amplify the target DNA sequence and is indicative of the fidelity of PCR. **Figure 2A** depicts an example of a typical CE separation of a series of six dsDNA fragments (markers) ranging in size from 50 to 1000 bp. To determine the size of specific PCR products, an aliquot of the PCR sample, containing a single **(Fig. 2B)** or two different **(Fig. 2C)** amplicons, is mixed directly with the dsDNA markers and subjected to CE using the conditions described in **Table 3**. Size of the PCR products can be extrapolated from a plot of marker size in bp vs migration time **(Fig. 2*d*)**. For amplicons ranging in size from 50 up to about 500 bp, linear regression analysis can be used to estimate the actual size of the dsDNA (Fig. 2d, inset). This approach assumes that amplicon size differs sufficiently from the marker peaks to ensure adequate resolution of both peaks. In cases where the amplicon size coincides with that of the marker peak, it would be necessary to employ a dual label system employing spectrally resolved dyes so that the co-migrating peaks could be discriminated ***(18)***. Another approach is to use customized sets of dsDNA markers (developed by

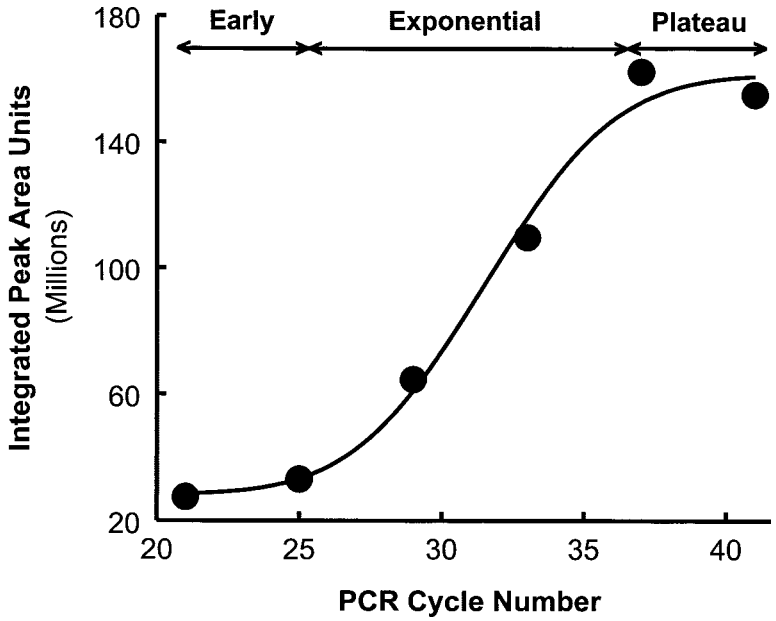


Fig. 1. A plot of amplicon integrated peak area fluorescence units vs thermal cycle number from the amplification of a 300 bp chicken cytoplasmic ß-actin gene sequence. RT-PCR was used to analyze 1 µg of total RNA isolated from 8 week-old male broiler chicken liver according to conditions listed in **Table 2**. Amplicons were separated and detected using CE/LIF as outlined in **Table 3**. Three ranges of the PCR amplification, early, exponential (linear) and plateau are indicated.

PCR or obtained from commercial sources) that "bracket" the anticipated size range of the amplicon peaks ***(19)***.

The ability to do on-capillary detection and to calculate integrated peak area makes CE/LIF very useful for the determination of dsDNA products generated by RT-PCR. The quantity, as determined by integrated peak area, of each amplicon is indicative of the efficiency of PCR. Such information is also useful in determining the amount of a specific mRNA present in the original RNA sample. However, to accurately quantify mRNA levels by RT-PCR, internal standards or controls must be included to normalize the data and to account for differences in RNA concentration and efficiencies of the RT and PCR steps ***(16)***. Co-amplification of exogenous or endogenous standard template sequences allows for the correction of variable product yields due to differences in individual efficiencies of RT and PCR and other sources contributing to tube-to-tube variation such as:

1. Inaccurate assessment of initial RNA concentration.
2. Differential yield of cDNA.
3. Differences in PCR product yield.
4. Pipeting errors.

With the use of appropriate standards, it is possible to obtain either a relative or an absolute estimate of mRNA level in conjunction with CE/LIF analysis of the PCR products. In the case of exogenous standards, these can either be synthetic RNA or dsDNA templates. Although exogenous dsDNA standards can be added at the PCR step to correct for tube-to-tube variation, using endogenous or synthetic RNA standards is advantageous because they correct for variation originating from both the RT and PCR steps.

3.2. Capillary Electrophoresis and Relative-Quantitative-RT-PCR

Relative-quantitative-RT-PCR (RQ-RT-PCR) compares the level of target sequence across a

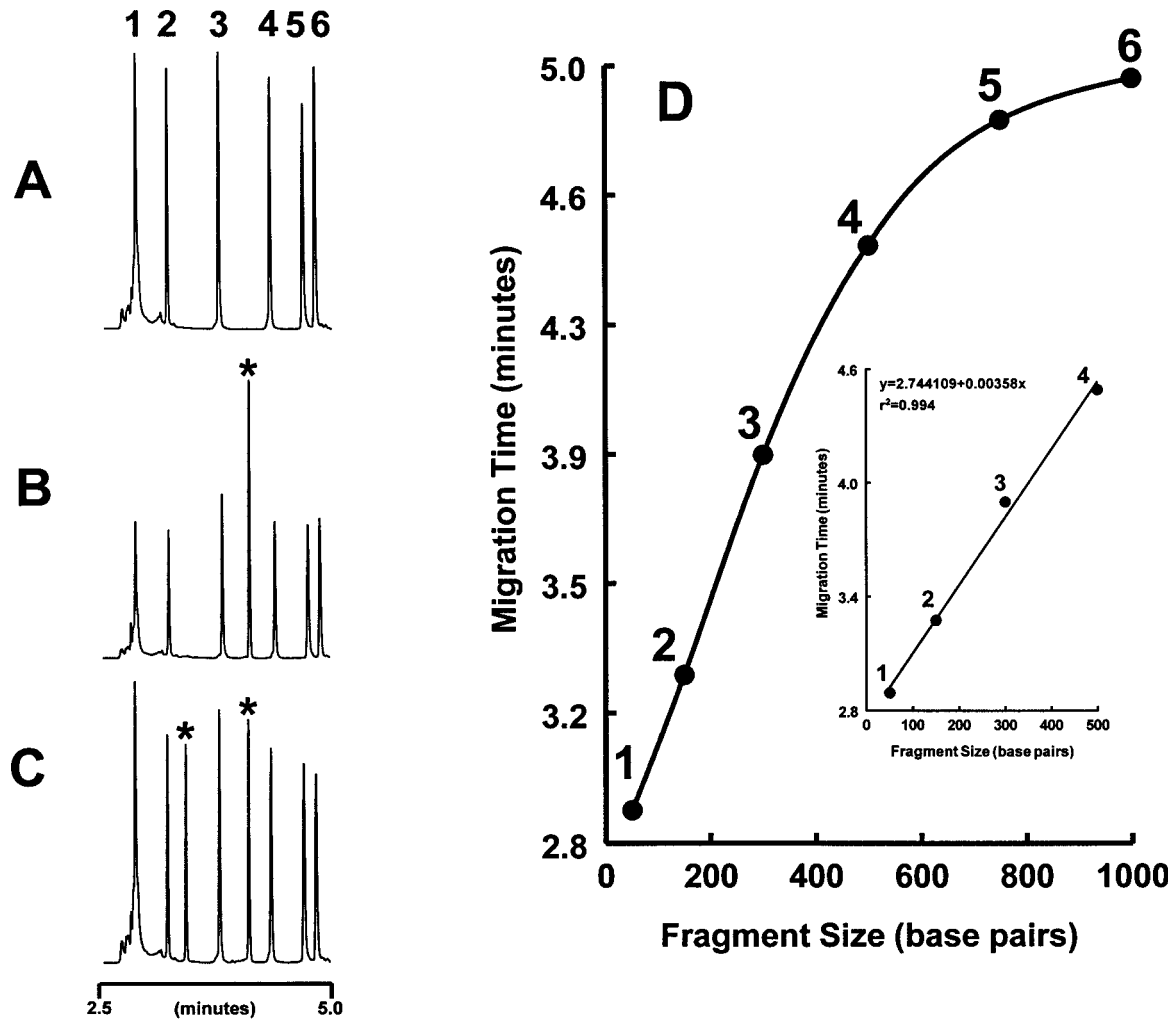


Fig. 2. Size analysis of dsDNA PCR products performed using CE/LIF. **(A)** A series of dsDNA markers (1–6= 50, 150, 300, 500, 750, and 1000 bp, respectively) was separated and detected by CE/LIF according to the conditions outlined in **Table 3**. The relationship between peak migration time (min) and dsDNA fragment size (bp) is depicted (D). The actual size of individual amplicons for pig leptin receptor **(B)** or IGF-1 and IGF-2 **(C)** was determined by the addition of the RT-PCR sample into a sample of the 6 PCR markers and determining the migration time of the amplicons (*) relative to that of the markers **(D)**. For amplicons less than 500 bp linear regression analysis can be used to determine amplicon size (**D**, inset). Using this approach, amplicon sizes of 400, 207, and 400 bp were determined for pig leptin receptor (predicted 398 bp), IGF-1 (predicted 202 bp) and IGF-2 (predicted 400 bp), respectively. It is possible to determine size of amplicons from multiplex RT-PCR **(C)** provided that all amplicon peaks can be resolved from the size marker peaks.

range of samples using a common co-amplified internal (endogenous) standard sequence for normalization ***(20)***. Results are expressed as a ratio of the analyzed level (integrated peak area) of target amplicon to that of an internal standard. Thus, a corrected or relative value for target gene sequence is produced for each sample subjected to RQ-RT-PCR, and this ratio value estimates the level of gene expression (mRNA level) relative to a common internal standard gene.

The appropriate choice of an internal standard for RQ-RT-PCR is crucial for a valid quantitative comparison of mRNA levels across samples. Ideally, the expression of an internal standard gene should be invariant across the range of tissues and treatments being analyzed. At the conclusion of RT-PCR, the accumulated level of the internal standard amplicon must fall within the exponential amplification range together with that of the target gene amplicon. This ensures that the rate of amplification was similar for both templates. Also, the primers used to amplify the target sequence must be compatible with those used to amplify the internal standard. They must not produce additional products or hybridize with each other ***(21)***. Target amplicon quantity is then normalized to the internal standard amplicon for reactions collected during the exponential phase of PCR amplification by dividing the quantity (integrated peak area units) of target by the corresponding value for the internal standard. If the rate of amplification is similar for both target and internal standard, then this ratio should remain constant and thus accurately reflect the mRNA ratio in the original RNA sample.

There have been reports evaluating the suitability of specific internal standards for quantitative analysis of mRNA levels ***(22,23)***. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ß-actin, and cyclophillin are a few of the constitutively expressed "housekeeping" genes that have been used for standardization of RQ-RT-PCR. Ribosomal RNA (rRNA), which comprises 80% of total RNA extracts, has also been utilized. It is thought to be much less variable than individual mRNAs under most experimental conditions because rRNA transcription employs a distinct RNA polymerase enzyme ***(23)***. However, 18S and 28S rRNA would not be appropriate internal standards for RT reactions primed with oligo dT or when poly A^+ RNA was used. Also, because of their high levels of expression, 18S and 28S rRNAs are not well suited to serve as standards for quantifying genes expressed at low levels.

Figure 3 demonstrates an RQ-RT-PCR analysis of chicken liver malic enzyme (EC 1.1.1.40) gene relative to three different co-amplified internal standards, ß-actin, GAPDH, and 18S rRNA. For the 18S rRNA standard, the use of competimer technology (Ambion Inc., Austin TX) is shown with respect to its ability to modulate the amount of 18S rRNA PCR product formed. By adding increasing amounts of blocked internal standard primers (competimers) that anneal to the template but cannot be extended, the amplification of the 18S rRNA template is attenuated during PCR. This attenuation shifts the exponential amplification range of PCR so that the 18S rRNA internal standard and target (malic enzyme) gene amplicons accumulate at similar rates. We routinely fix the size of the internal standard amplicon (e.g., 300 bp) so that the size of the target amplicons can vary relative to the standard. This is particularly useful in developing RQ-RT-PCR assays for expression of multiple (more than two) genes in what is commonly referred to as a "multiplexed" format.

Figure 4A depicts the application of RQ-RT-PCR to the analysis of metallothionein (MT) gene expression in isolated hepatocytes obtained from turkey embryos and cultured in medium containing 10% fetal bovine serum. Metallothionein is a low molecular weight heavy metal binding protein thought to play a role in intracellular metal metabolism ***(24)***. MT is unique in that metals that bind to the protein also induce expression of the gene. The effect of zinc (100 μM) added to the culture medium on MT gene expression was assessed over time (0–24 h). The assay consisted of a duplex reaction with co-amplification of MT and ß-actin internal standard templates. **Figure 4B** depicts a plot of the change in MT/ß-actin inte-

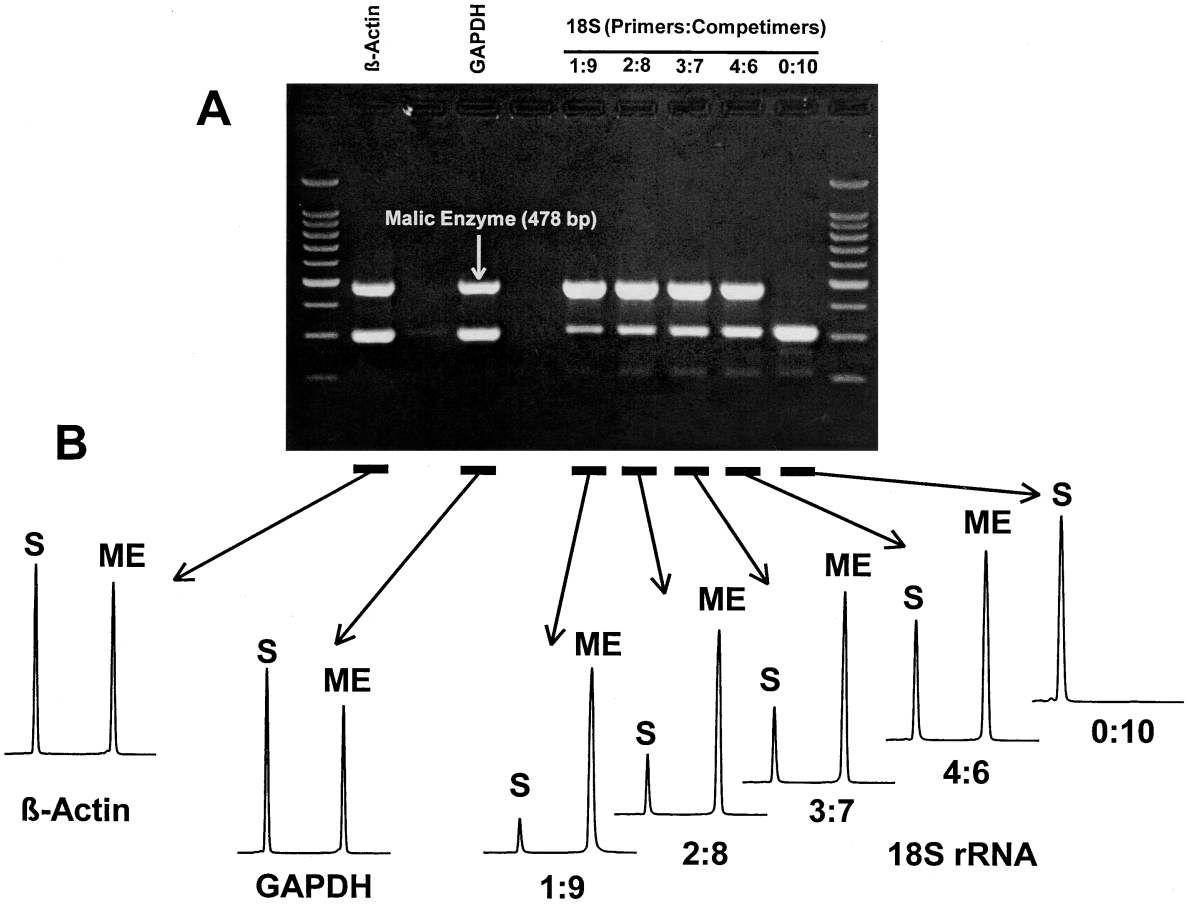
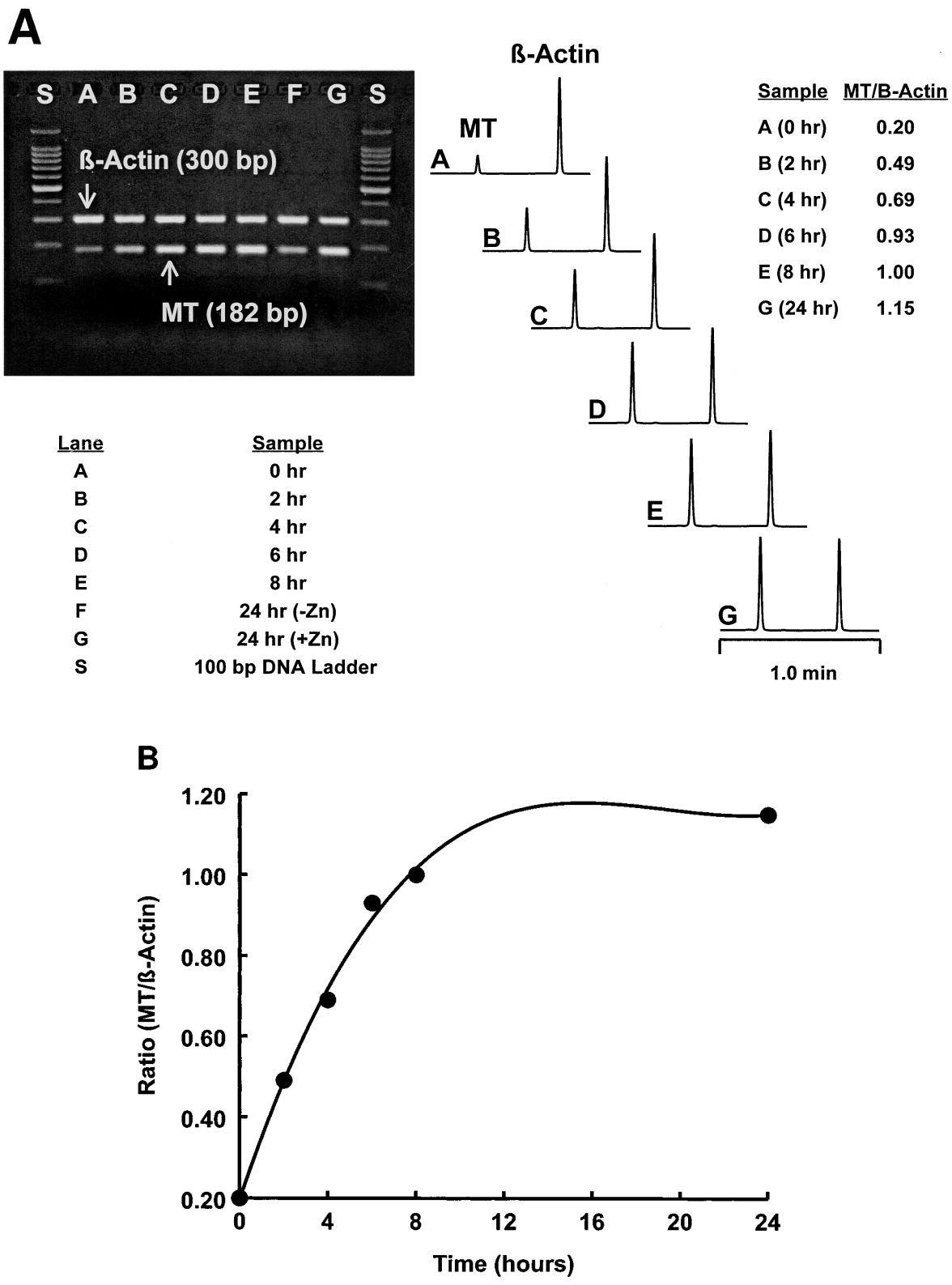


Fig. 3. Duplex RT-PCR using three different internal standard controls (S) for the relative quantitation of chicken liver malic enzyme (ME) gene exprression. Glyceraldehyde phosphate dehydrogenase (GAPDH), ß-actin and 18S rRNA were co-amplified along with ME. Samples were separated by agarose (2%) slab gel electrophoresis with ethidium bromide staining **(A)** and by CE/LIF **(B)**. The amplification of 18S rRNA involved the use of a mixture of gene specific primers and blocked primers that cannot be extended in PCR (competimers, Ambion Inc, Austin, TX). By adjusting the ratio of primers to competimers, it is possible to control the amount of PCR product for this gene and thereby adjust it to match the level of product for the gene being quantified (ME). Competimer:Primer ratios of 1:9, 2:8, 3:7, 4:6 and 0:10 were evaluated with respect to the production of an 18S rRNA amplicon (315 bp).

grated peak area ratio over time and indicates a rapid rise in MT gene expression in response to the added zinc, reaching an upper plateau between 6 and 24 hr. This example emphasizes the point that it is not always necessary to obtain absolute estimates of mRNA levels. Instead, relative estimates obtained under carefully controlled conditions can provide an excellent indication of gene transcriptional activity in response to an experimental treatment or changing physiological state. Moreover, RQ-RT-PCR coupled with CE/LIF provides for a rapid and accurate analysis of the numerous samples that are typically generated by such experimental designs.

Figure 5 depicts the results of a multiplexed analysis in which the expression of two different genes (insulin-like growth factor I, IGF-1; and insulin-like growth factor II, IGF-2) is compared to that of a common internal standard (ß-actin). This approach affords several advantages over


A
S A B C D E F G S
ß-Actin (300 bp)
MT (182 bp)
ß-Actin
MT
A
B
C
D
E
G
1.0 min
Sample MT/B-Actin
A (0 hr) 0.20
B (2 hr) 0.49
C (4 hr) 0.69
D (6 hr) 0.93
E (8 hr) 1.00
G (24 hr) 1.15
Lane Sample
A 0 hr
B 2 hr
C 4 hr
D 6 hr
E 8 hr
F 24 hr (-Zn)
G 24 hr (+Zn)
S 100 bp DNA Ladder
B
Ratio (MT/ß-Actin)
1.20
1.00
0.80
0.60
0.40
0.20
0
4
8
12
16
20
24
Time (hours)

conducting individual analyses to estimate the expression of either gene separately. First, using a common internal standard allows for a more accurate direct comparison of the level of gene expression of IGF-1 relative to that of IGF-2. This approach would be particularly useful for the comparison of expression levels of genes that are linked by common induction mechanisms or are coordinately expressed. Second, the use of a single reaction for multiple estimates of gene expression presents considerable cost savings for reagents and time. It is important to keep in mind that multiplexing presents a number of challenges that must be met including:

1. Optimization of reaction conditions to ensure constant rates of amplification for all templates.
2. An appropriate choice of primer sets that do not interact with each other.
3. All templates must be amplified independently of each other.
4. Appropriate adjustments to reagents and conditions to ensure optimal product yields.
5. An appropriate choice of amplicon size (as dictated by primer sets) to ensure adequate resolution of all amplicons in post-PCR CE/LIF separations ***(21,25)***.

Others have reported using multiplexed RQ-RT-PCR in conjunction with CE/LIF for the relative quantitation of gene expression ***(26)***. In some cases, multiplexing of RQ-RT-PCR reactions does not permit all target templates to be amplified at similar rates because of markedly different expression levels of the genes being studied. To compensate for this, a technique referred to as "primer dropping" can be employed in which primer pairs are added at different points during PCR to compensate for the different levels of target template abundance ***(26)***. Effectively what this does is to "adjust" the rates of amplification to similar levels so that all amplicons would be expected to fall within the exponential range upon completion of PCR. Thus, it is still possible to multiplex RQ-RT-PCR assays and obtain meaningful quantitative estimates for genes that are expressed at markedly different rates. However, care must be taken to determine optimal conditions for such an approach and to avoid contamination because of the required opening and closing of the PCR tubes during the reaction ***(21,25)***.

3.3. Capillary Electrophoresis and Competitive-RT-PCR

Accurate quantitation of gene expression can also be achieved by RT-PCR using a competitive approach. Quantitative-competitive RT-PCR (QC-RT-PCR) involves the addition of known amounts of an exogenous standard synthetic RNA (competitor) to samples containing an unknown amount of endogenous target mRNA prior to initiating RT-PCR ***(16,27–32)***. The competitor's sequence is most often designed to be nearly identical to that of the target except for a small (,10%) addition, deletion or other modification of sequence. Such modification allows the competitor and target amplicons to be similar enough so as to be amplified at the same rate in PCR, but sufficiently different in size to be separated and accurately quantified during post-PCR electrophoretic analysis. The competitor and target must share identical primer annealing sites for PCR amplification. Thus, a competition develops for primers, dNTPs and Taq between the two templates during PCR. Assum-

Fig. 4. RT-PCR analysis of metallothionein (MT) gene expression by hepatocytes obtained from day 16 turkey embryos and cultured in medium containing 10% fetal bovine serum. The effect of supplemental zinc (100 μM) added to the culture medium on MT gene expression was determined over time (0–24 h). Duplex RT-PCR was performed and MT gene expression was determined relative to that of ß-actin **(A)**. PCR products were separated by agarose (2%) slab gel electrophoresis with ethidium bromide staining and by CE/LIF. The ratio of integrated peak area fluorescence units for MT and ß-actin was used to determine relative level of MT gene expression. The MT/ß-actin peak area ratio was plotted as a function of time following addition of zinc **(B)**. This plot demonstrates an initial (0–6 h) rapid rise in MT gene expression by the cultured hepatocytes in response to supplemental zinc that reached an upper plateau between 6 and 24 h following exposure to the metal.

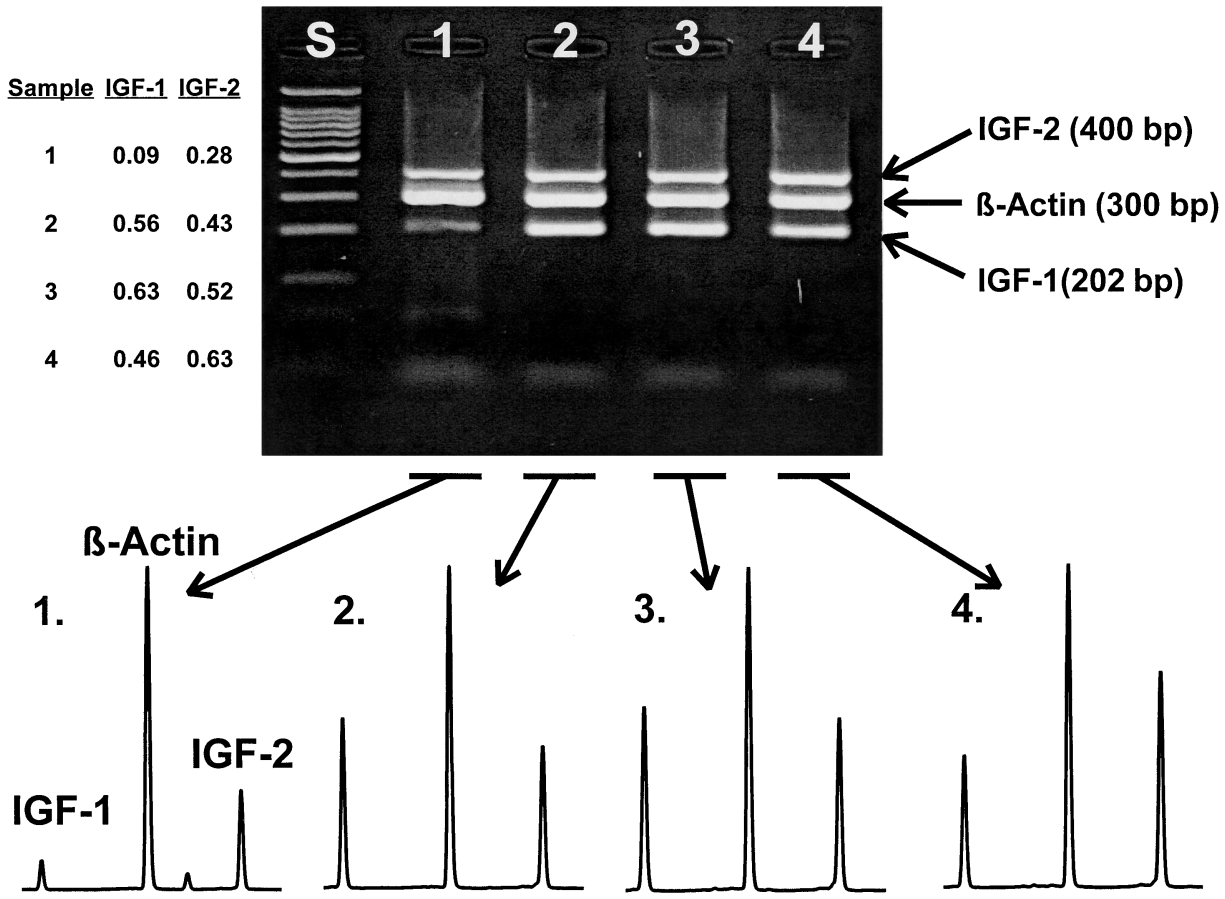


Fig. 5. An example of a multiplexed RQ-RT-PCR analysis to determine the expression of insulin-like growth factor 1 (IGF-1) and insulin-like growth factor 2 (IGF-2) relative to a common co-amplified ß-actin internal standard. Total RNA was obtained from liver tissue collected from a 16-day-old turkey embryo **(1)**, a 3-wk-old turkey poult **(2)**, a 3-wk-old male broiler chicken **(3)** and a 32-wk-old female broiler egg-laying chicken **(4)**. PCR products were separated by agarose (2%) slab gel electrophoresis with ethidium bromide staining and by CE/LIF. The peak area ratios of the two IGF genes relative to ß-actin calculated from the CE/LIF analysis are shown demonstrating the changes in IGF gene expression for the different samples analyzed.

ing that the competitor and target templates are amplified at the same rate, the ratio of the two PCR products should reflect the initial ratio of the two templates in the original RNA sample. Since the absolute amounts of competitor added to the RNA sample are known, the amount of target mRNA can be determined.

A number of methods have been reported for the design of synthetic RNA competitors ***(16,27–32)***. Figure 6 depicts a procedure that we have used to create a synthetic RNA competitor for QC-RT-PCR analysis of leptin gene expression in chickens ***(33,34)***. Our approach made use of restriction enzyme digestion to excise a small (27 bp) fragment from a 509 bp chicken leptin clone containing the entire coding region of the gene and ligation of the remaining two larger pieces (222 and 286 bp) to create a competitor sequence that is 27 bp shorter in length than the target sequence. This sequence was subcloned into an appropriate expression vector and a synthetic leptin competitor RNA transcribed in vitro. In fact, this particu-

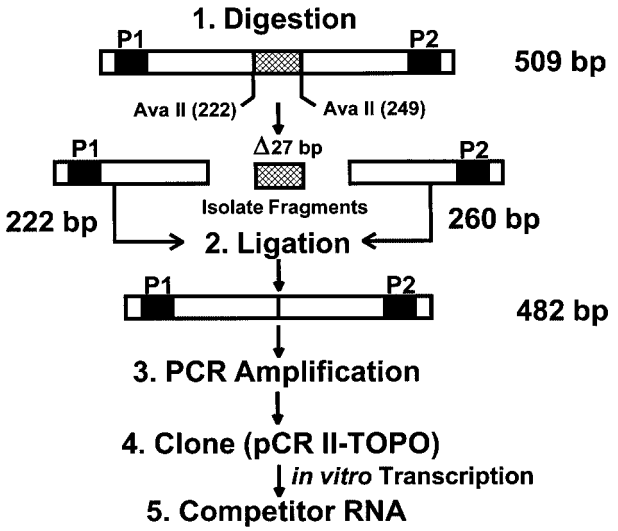


Fig. 6. Procedure used for the construction of a chicken leptin internal synthetic RNA competitor by excision of a 27 bp segment from a cloned DNA fragment (509 bp) containing the full-length coding region for the chicken leptin gene. To generate the competitor construct, the following steps were performed: 1. restriction enzyme digestion with *AvaII*, 2. ligation of the resulting 222 bp and 260 bp restriction fragments, 3. PCR amplification of the 482 bp product of the ligation step, and 4. cloning of the PCR-amplified 482 bp dsDNA product into an appropriate expression vector (pCR 2.1-TOPO). Both the target and competitor share two unique primer recognition sites (P1 and P2) that are highly conserved across a number of vertebrate species. Using P1 (5´-TGACACCAAAACCCTCATCA-3´) and P2 (5´-GCCACCACCTCTGTGGAGTA-3´) in PCR produced a target amplicon of 348 bp and a competitor amplicon of 321 bp.

lar competitor sequence was designed to contain two internal primer recognition sites (P1 and P2) that are highly conserved across vertebrate species. This enabled us to utilize the same competitor (designed from chicken leptin gene sequence) for QC-RT-PCR analyses of leptin gene expression across species. In effect, this construct serves as a "universal" competitor for QC-RT-PCR analysis of leptin gene expression.

Figure 7 demonstrates the approach taken to estimate the level of leptin mRNA contained in a total RNA sample isolated from human placental tissue with the "universal" competitor standard. Using QC-RT-PCR and CE/LIF, it was possible to derive an absolute estimate (typically expressed as attomoles or copies per microgram total RNA) of leptin mRNA. By plotting the log of different competitor/target peak area ratios against the log of the amount of added competitor and extrapolating from the point at which the ratio is equal to one (i.e., log target/log competitor=0), the amount of leptin mRNA in the original sample can be determined.

There have been a number of reports demonstrating a variety of applications of QC-RT-PCR and CE/LIF for the quantitative analysis of gene expression ***(10,35–37)***. Others have demonstrated the possibilities for multiplexing QC-RT-PCR in which more than one target/competitor pair is subjected to co-amplification with subsequent analysis of the products by CE/LIF ***(35,37)***. With multitarget QC-RT-PCR it is possible to monitor PCR amplified dsDNA corresponding to several genes simultaneously in a single sample assuming PCR conditions have been optimized for each product formed ***(21,25)***. Unique competitors have been developed specifically for use with QC-RT-PCR in multiplexed reactions. Theses competitors contain contiguous sequence and primer sites for multiple genes all linked together ***(29,38,39)***. This allows a single competitor to be added to reactions for the analysis of multiple genes. For example, Jensen and Whitehead ***(39)***constructed a synthetic RNA transcript from 10 oligonucleotides that they refer to as a "polycompetitor" for use in multiplexed QC-RT-PCR to simultaneously study changes in the expression of acute phase protein genes coding for serum amyloid A, serum amyloid P component, C-reactive protein, apolipoprotien A1, apolipoprotein A2, GAPDH, and ß-actin. Although this approach greatly simplifies the assembly of reaction tubes for QC-RT-PCR, precautions must be taken to optimize multiplexed reactions ***(21,25)***.

4. Future Developments

Future applications of CE to RT-PCR product analysis will arise from refinements and improve-

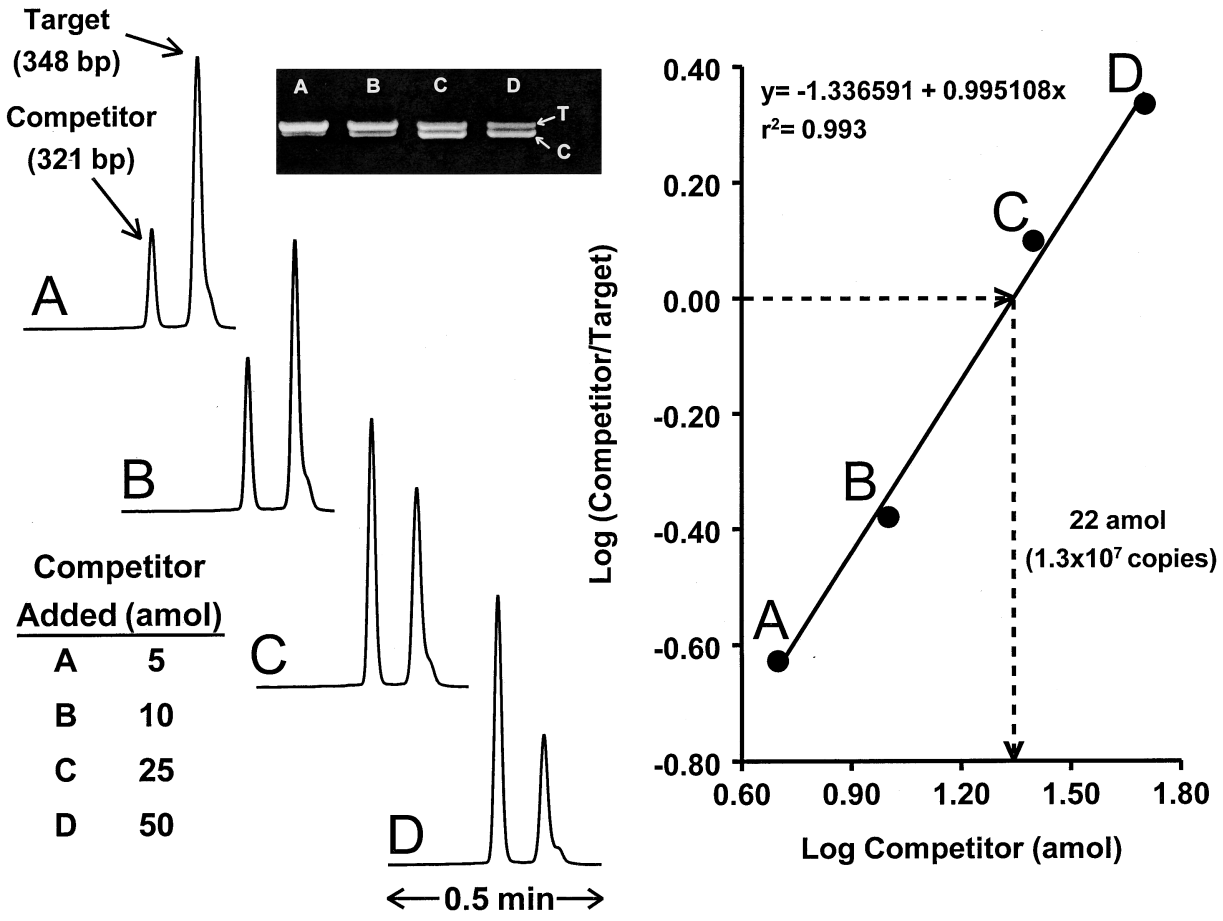


Fig.7. Using QC-RT-PCR to determine the absolute amount of leptin mRNA in a human placental tissue total RNA sample. Four levels of a synthetic RNA competitor (A-D; 5, 10, 25, 50 amol) were added to 4 separate assay tubes each containing 1 μg of total RNA. The dsDNA amplicons produced by PCR were separated and detected by CE-LIF. A portion (1 min, corresponding to 4-5 min of the 5 min run) of each of the 4 electropherograms is depicted on the left side of the figure with the target (348 bp) and competitor (321 bp) amplicons denoted. Also shown is a portion of the PCR sample subjected to agarose (2%) slab gel electrophoresis with ethidium bromide staining to separate the target (T) and competitor (C) amplicons. Integrated peak areas for both amplicons were determined from the CE/LIF data and a competitor/target peak area ratio of was determined. The log of the target/competitor peak area ratio was plotted against the log of the quantity of added synthetic competitor RNA (right) and the quantity of leptin mRNA in the sample (22 amol or 1.3x107 copies/μg total RNA) determined from the regression equation shown at the point where the log of the ratio was equal to zero (i.e., target=competitor).

ments in CE instrumentation. Advances in miniaturization of CE devices involving electrically driven microfluidics are already contributing to the next generation of instrumentation. Ultra-fast separations of DNA performed on glass or plastic chips with etched micro-channels have proven to be feasible ***(40–43)***. Chips onto which a large number of channels can be etched or capillary arrays are both potential approaches for increasing sample throughput in CE. The integration of robotics with analytical instrumentation to facilitate sample preparation and loading prior to RT-PCR and/or CE/LIF is another way of increasing sample throughput. Incorporation of multiple cap-

illaries or capillary arrays into automated DNA sequencing instruments has greatly reduced analysis time and increased sample throughput and this development have played a crucial role in accelerating sequencing of the human genome ***(44)***. Addition of emerging fraction collection technology to commercial CE instruments will greatly expand the functional capability of such instruments by allowing for post-separation analyses such as cloning, sequencing or reamplification ***(45,46)***. Coupling of new detection methods such as electrochemical detection ***(47)*** to CE devices as well as refinements in existing detection methodology such as LIF offer the promise of substantial increases in selectivity, detection sensitivity and more accurate quantification of PCR products.

Development of fully integrated devices that combine fast electrophoretic separation methods with microscale sample preparation and processing are key features for developing new instrumentation and capabilities. Recently, it has been shown to be possible to integrate PCR amplification and CE separation of PCR products in a single device using an array of eight capillaries ***(48)***. Similarly, attempts have been made to develop chips on which PCR and electrophoretic separation of the amplified DNA sequences are performed consecutively ***(49,50)***. This type of technology could be used to produce dedicated instruments ("lab-on-a chip") for gene expression analysis that would feature rapid run time and high throughput. It may even be possible to integrate a number of steps involved in RT-PCR including: cell lysis, mRNA isolation and enrichment, RT-PCR and fast electrophoretic separation of PCR products, all in a single self-contained device that relies on electrically-driven microfluidics to move sample and reagents around as well as to drive the separation of dsDNA PCR products ***(49,51)***. In fact, a fully integrated chip-based device has been developed that utilized microfabricated fluidic channels, heaters, temperature sensors, and fluorescence detectors for DNA analysis following amplification or enzymatic digestion ***(52)***.

RT-PCR and CE/LIF could also be utilized in conjunction with orthogonal analytical methods for assessing gene expression. For example, hybridization microarray technologies may benefit from a more in-depth study of candidate gene expression with estimates determined separately and confirmed by RT-PCR and CE/LIF. Microarray techniques would serve as an initial screening tool whereas RT-PCR with CE/LIF would be used to produce more accurate and precise quantitative estimates of candidate gene expression. There has also been some exploration of adapting CE/LIF with fraction collection capability to differential display analysis for the purpose of characterizing novel gene expression events ***(53,54)***. If the technical challenges can be solved, this approach would greatly enhance a comparative approach to studying differential gene expression.

An increasing number of clinical and agricultural testing uses for RT-PCR and CE/LIF are likely to be identified in the near future, as monitoring of gene expression becomes more of a routine diagnostic tool. Fully integrated analytical devices will undoubtedly further accelerate RT-PCR/CE-LIF technique development and application. Newly emerging fields such as molecular medicine and pharmacogenomics will increasingly require accurate quantitative assessments of gene expression in addition to information about gene sequence and genomic organization. For example, CE/LIF has been utilized in conjunction with fixed paraffin-embedded tissues to perform quantitative RT-PCR to estimate gene expression, making this approach potentially very useful for biomedical research and epidemiological studies ***(55)***. The combination of RT-PCR and CE/LIF offers a great deal of potential for rapid and quantitative analysis of a variety of mRNA transcripts from many samples, especially when using a multiplexed format.

5. Conclusions

The combination of RT-PCR with its high sensitivity for mRNA detection and amplification and CE/LIF with its power to rapidly separate and detect very small amounts of dsDNA provides an efficient method to quantitatively analyze gene expression. It is particularly well suited to the analysis of samples from limited supplies of tis-

sues or cells as well as genes that are expressed at low levels. CE coupled to LIF provides an attractive alternative to conventional slab gel methods. With our expanding knowledge of genomics has come an enhanced demand for expression analysis of larger sets of genes. However, techniques such as array hybridization that can quantify expression levels of a large number of genes simultaneously are still under development and their estimates may lack sufficient sensitivity and precision. The automated features of commercial CE instruments permit rapid analysis and high sample throughput. Either relative or absolute estimates of mRNA levels derived from this technique allow researchers to study the gene expression more quickly and more accurately. In the future, it is anticipated that CE-based analysis of gene expression will continue to get faster and more reliable as it gains wider acceptance.

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